General introduction
Ubiquitin, Signaling, and Cancer

The orchestration of all cell biological processes requires intensive and continuous communication. This becomes apparent in many diseases, where the underlying defects consist of perturbations in specific signaling networks that fail to generate the desired physiological output. For many pathologies, including cancer, malfunctioning is caused by genetic alterations that render specific systems either unresponsive, hyper-active or simply nonfunctional.

Protein regulation

The primary mediators of signaling networks and pathways are proteins and their activity is regulated at almost all thinkable levels. Indeed, in malignant cells many examples of genetic alterations have been described that affect one or multiple of these regulatory processes[1]. Protein regulation can be divided into pre- and post-translational processes.

The starting point of all protein regulation is gene transcription. The importance of transcriptional regulation is illustrated by the fact that more than 10% of the proteins encoded in the human genome are transcription factors[2]. Together these proteins regulate the number of mRNA copies that are transcribed from the 22.000 or so genes present in every cell. In higher eukaryotes, subsequent alternative splicing of mRNAs contributes to the next level of complexity. Recent data indicates that at least 74% of all human multi-exon genes are alternatively spliced[3]. Furthermore, it has been estimated that 15% of the single point mutations that cause human diseases affect alternative splicing[4].

More recently, the discovery of a large number of small hairpin RNAs that function to inhibit translation, so called micro-RNAs (miRNAs), suggests that the regulation of protein abundance at the level of translation may play a more important role than previously anticipated. Estimates now indicate that up to 30% of all transcripts may be regulated by over 1000 distinct miRNAs, uncovering an unanticipated network of small regulatory RNAs[5,6].

Ubiquitin: a truly ubiquitous protein

Once translation has been completed and proteins have been properly folded, most proteins are subject to a diverse range of post-translational modifications that influence their function. Many proteins are differentially hydroxylated, methylated, acetylated, phosphorylated or conjugated to ubiquitin and ubiquitin-like (Ubl) polypeptides. Over the last few decades, protein modification by ubiquitin and Ubl molecules has emerged as a central regulatory process in virtually all aspects of cell biology[7,8]. More than 10 different ubiquitin and Ubl modifications have been described (see Table1)[9,10]. Futhermore, under standard conditions in yeast, up to 20% of all proteins are conjugated to ubiquitin and more than 270 proteins are modified by the Ubl molecule SUMO[10-12].

The biochemical processes underlying ubiquitination of proteins have been studied in great detail. An enzymatic cascade carried out by three, or sometimes four, different classes of proteins (E1, E2, E3 and E4) serves to conjugate ubiquitin to a target[13]. The first step in ubiquitination is the generation of a highly reactive thioester bond between the E1, also termed ubiquitin activating enzyme, and a glycine residue at the C-terminus of ubiquitin. Next, the activated ubiquitin is transferred to an E2. Often a single E2 enzyme is involved in the regulation of multiple proteins in a common pathway. Finally, interaction of the E2 with an E3 bound to a substrate, catalyses conjugation of the ubiquitin moiety onto a lysine of the target protein. In some cases, a fourth enzyme (E4) appears to be required to catalyze the generation of a poly-ubiquitin chain.

The selective interaction of the E3 with the substrate determines specificity. E3s, also called ubiquitin ligases, make up one of the largest protein families in the human genome, rivaling the protein kinase family[14]. More parallels can be drawn between protein phosphorylation and ubiquitination. Like protein phosphorylation, ubiquitination is reversible. The human genome contains more than 60 deubiquitinating enzymes (DUBs) that cleave ubiquitin moieties from target proteins. A more thorough discussion of these enzymes is given later in this introduction. In addition, protein ubiquitination is fast: ubiquitination can take place within minutes after stimulation. Lastly, analogous to the phospho-protein interaction domains SH2 and PTB, specialized protein modules have evolved that allow interaction with specific forms of ubiquitin (see table 2) [15].

Table 1 Ubiquitin and Ubiquitin like modifications

Name	Function/process	Substrate examples	Remark(s)
K48 poly-ubiquitin	Proteasomal degradation	Many (cell cycle) proteins, HIF1α, SMADs	
Monoubiquitin	DNA-repair, receptor internalization, transcription	PCNA, FANCD2, BRCA2, EGFR, Histones	
K63 poly-ubiquitin	Kinase activation, DNA repair, transcription	NEMO, PCNA, TRAFs, RNA-pol II	
K6 poly-ubiquitin	IKK activation, DNA repair?	NEMO	BRCA1/BARD and cIAP catalyze K6 Ub-chains
SUMO1-3	Nuclear-cytoplasmic localization, DNA repair, transcription	RanGAP1, Iκ-Bα PML, PCNA, MDM2, CtBP1	UBC9 only known SUMO E2
NEDD8	SCF ligase activation, checkpoint signaling	Cullins, p53	
ISG15	Adaptive immune response	JAK1, ERK1	Deconjugated by USP18
LC3/APG12p	Autophagy, microtubule dynamics	Unknown	
Hub1p	Polarization and mRNA splicing in yeast	Spt1p, Hbt1p	No human homologue identified
FAT10	Apoptosis	Unknown	TNF-α target
Urm1p	Budding and nutrient signaling in yeast	Ahp1p	No human homologue identified

The Proteasome: a central regulator

The most studied and best understood role of ubiquitin involves the targeted destruction of proteins by the 26S proteasome. Whereas protein synthesis is relatively slow, only allowing dampened responses, protein degradation is fast and efficient. Therefore, the steady-state levels of proteins that must undergo fast and dramatic changes in abundance are often regulated by active protein degradation. The covalent attachment of at least 4 ubiquitin moieties, linked through the lysine at position 48 of ubiquitin itself (K48), to a single lysine on the target protein serves as a tag to send the protein for destruction by the 26S proteasome. A critical role for the proteasome has been described in virtually all aspects of cancer. Aberrant proteolysis of proteins has been linked to defects in apoptosis, the cell division cycle, DNA damage signaling and gene transcription[16-18]. Furthermore, the observation that many cancer cells are more susceptible to proteasome inhibition than normal cells has sparked intensive research into applications of proteaseome inhibitors in the cancer clinic. Currently, the inhibitor bortezomib (Velacade) is approved for the treatment of multiple myeloma, and other applications are under study[19,20]. However, the mechanism(s) responsible for the increased sensitivity of cancer cells remain poorly understood.

In some cases, specialized proteins that recognize multi-ubiquitin chains on poly-peptides are required for efficient proteasome targeting[21]. Rpn10 and Rad23 are two ubiquitin interaction domain-containing proteins that associate with the proteasome (also see Table 2). Interestingly, Rpn10 and Rad23 both bind and contribute to the degradation of Sic1 (a yeast CDK inhibitor) and Clb2 (an APC substrate). In contrast, the degradation of Far1 (another yeast CDK inhibitor) does not depend on Rpn10, but instead on Rad23 only. Furthermore, other proteasome substrates do not rely on either protein. Since Rad23 and Rpn20 require both their proteasome interaction and ubiquitin binding domains, this suggests that proteasome interacting molecules promote the degradation of specific target molecules, adding an additional layer of substrate specificity.

The proteasome is sometimes referred to as the cellular garbage disposal machine and literally grinds up proteins into short peptides but at the same time recycles the intact ubiquitin molecules[22]. The proteasome is a labile structure that can disassemble in vitro into a 20S and 19S particle. Structural studies indicate that the 20S particle forms a cylindrical shaped multiprotein complex with an estimated channel diameter of 13 Å[23,24]. The proteolytic sites of the proteasome face the interior of this cylinder, to which access is restricted

Table 2 Ubiquitin interaction domains. Adapted from Schnell and Hicke[15] and ENSEMBL and Interpro databases

Domain	Example(s)	Size (amino acids)	Remarks
UEV	MMS2/UEV1a	~145	Involved in K63 ubiquitin conjugation
NZF	Npl4, Vps36	~35	
ZnF UBP	HDAC6, various deubiquitinating enzymes	~58	Also known as PAZ domain
UBA (ubiquitin associated domain)	RAD23, CBL, USP13, USP5, LATS	45-55	
UIM (ubiquitin interaction motif)	PSD4/Rpn10, MEKK1, USP25, USP37, Ataxin3	~20	
CUE	TOLLIP, TAB2, TAB3	42-43	K63 poly-ubiquitin specific?

by the 19S regulatory particles. The limited diameter of the proteasome suggests that proteins need to be stripped from their branching ubiquitin chains and unfolded prior to entry into the proteasome. Indeed, deubiquitinating enzymes present at the lid of the proteasome are required for proteins to be degraded[25,26]. Finally, the short peptides generated by the proteasome are substrates for MHC class I molecules, and thus used to present intracellular antigens to the immune system[27].

Other emerging roles for ubiquitin

As mentioned, protein ubiquitination comes in many other flavors than K48 poly-ubiquitination, and serves more functions than protein degradation. Monoubiquitination of proteins, for example, has been reported to regulate vesicle sorting, viral budding, receptor internalization, gene transcription and DNA repair[28,29]. Besides monoubiquitination, different poly-ubiquitin branches have been described. In yeast, it was found that all seven lysines of ubiquitin can be used as branching sites (lysines 6, 11 27, 29, 33 48 and 63)[11]. The relevance and function of most of these different types of branches remain unknown, but proteasomal targeting of physiological substrates appears to be exclusively associated with K48 ubiquitin chains. These studies further indicated that K48 ubiquitination is the most abundant ubiquitin chain, followed by K63 ubiquitination. K63-linked poly-ubiquitin chains differ remarkably from K48 chains in their three dimensional structure, providing a mechanism for their different functions[30]. Although in yeast K63 ubiquitination is not essential, strains expressing a mutant form of ubiquitin that cannot form K63 chains (K63R mutant) have defects in diverse signaling systems. K63R mutant strains have been reported to display elevated sensitivity to temperature stress and defects in DNA repair, mitochondrial DNA inheritance, vesicle sorting, endocytosis of plasma membrane proteins, and ribosomal function[31-38]. Recent advances shedding light on the functions of monoubiquitination and K63 poly-ubiquitination have mostly come from studies on NF-κB signaling and DNA repair. Therefore, these two processes and the role of ubiqutin in particular, will be discussed in the following two sections.

Ubiquitin, NF-κB signaling, and cancer

NF-κB represents a group of structurally related transcription factors containing five mammalian members (c-Rel, RelA, RelB, p50 and its precursor p105, and p52 and its precursor p100) (reviewed in[39]). NF-κB is best known for its role in the immune response and as a regulator of apoptosis. However, deregulated NF-κB has been linked to almost all aspects of malignancy including telomere maintenance, angiogenesis and metastasis[40]. Iκ-B proteins retain NF-κB in the cytoplasm, thereby preventing nuclear translocation and transcription of target genes. A role for the proteasome in the activation of NF-κB has been well established. Upon activation of the pathway, phosphorylation of the Iκ-B proteins by a large kinase complex, termed the IKK, marks them for recognition and subsequent K48 poly-ubiquitination by the E3, βTRCP. Destruction of Iκ-B exposes a nuclear localization signal on NF-κB allowing nuclear import and binding to target promoters. Interestingly, Iκ-Bα contains a nuclear export signal and is an NF-κB target gene. In the presence of continuous IKK activity, this allows the generation of waves of NF-κB going in and out the nucleus[41,42].

More recently, K63 poly-ubiquitination has been implicated in activation of the pathway. The involvement of activating K63-chains was first recognized by Deng and colleagues[37]. In a series of elegant *in vitro* experiments they showed that both UBC13 and MMS2 (also known as UEV1a) are required for activation of the IKK. UBC13 and MMS2 form a heterodimeric E2 capable of mediating K63 ubiquitination. They went on to show that K63 ubiquitinated TRAF6 is capable of activating TAK1, a kinase known previously to phosphorylate and activate the IKK and MKK6[43]. Furthermore, TRAFs themselves are ring finger E3s, catalyzing the observed ubiquitination. Most likely this is not auto-ubiquitination, since TRAFs are found to oligomerize after stimulation of the NF-κB pathway by various stimuli[44]. Interestingly, two proteins that interact with the IKK, termed TAB2 and TAB3, contain ubiquitin interaction domains, suggesting a mechanism for recruitment of K63-TRAFs to the IKK (see also Table 2). Indeed, recent experiments indicate that TRAF recruitment via the TAB2/3 CUE domains is a critical step in the activation of the IKK[45].

TRAFs are not the only IKK activators to be modified by K63 ubiquitin chains. Both K63 and K48 ubiquitin chains regulate a second important regulator of IKK activation, named RIP. Remarkably, a single protein (A20) can cleave off the K63 chain and replace it with a K48 poly-ubiquitin chain. Is so doing, A20 not only inhibits activated RIP but also tags it for proteasomal destruction[46].

Since K63 ubiquitinated TRAFs are not degraded by the proteasome, their continuous presence would prevent transient IKK activation. Indeed, three independent reports, including one in this thesis, suggest that TRAFs are inactivated by deubiquitination[47-49]. A protein encoded by the familial tumor suppressor gene CYLD likely performs this task. In humans, mutations in CYLD lead to the development of a rare condition known as Cylindromatosis[50]. Patients suffering from this disease develop numerous benign skin tumors, mostly on the hairy areas of the body. The tumors, called cylindromas, are thought to arise from hair follicle cells and tumors show loss of heterozygosity of the CYLD locus. In agreement with an inhibitory role of CYLD in NF-κB signaling, mouse models indicate that NF-κB activity in these cells is essential for survival[51,52]. CYLD interacts with both the structural IKK component NEMO (also known as IKKγ) and TRAF2 suggesting that CYLD functions to inactivate TRAFs bound to the IKK by deubiquitination[47-49]. This is supported by the observation that both inhibition of CYLD by RNAi or overexpression of catalytically inactive CYLD mutants enhance TRAF ubiquitination and hyper-activate the IKK. Furthermore, like A20, CYLD is an NF-κB target gene, further indicating an important role for deubiquitination in inactivating the pathway[53,54].

The observation that in the absence of CYLD the IKK is hyper-activated, indicates that cylindromas may arise due to a failure of hair follicle cells to undergo apoptosis. Furthermore, this suggested that inhibition of the IKK could restore aberrant NF-κB activation and apoptosis. Indeed, in vitro studies showed that inhibition of IKKβ (one of the two IKK kinases) by salicylate restored the apoptotic response induced by TNF-α in cells lacking CYLD[49]. More importantly, this suggested a potential non-invasive therapy for cylindromatosis. Unpublished results now show that treatment of cylindromas with topical salicylic acid can inhibit and sometimes reverse tumor formation (Oosterkamp et al., submitted).

As mentioned, TAK1 also activates MMK6, a kinase involved in stimulation of the JNK pathway. Indeed, CYLD inhibition also enhances JNK activity after various stimuli (Nijman and Bernards, unpublished observation, and[55]). However, relevance of JNK activation in the pathogenesis of cylindromatosis remains to de determined.

Besides TRAFs and RIP, NEMO is also modified with ubiquitin chains other than K48, and this is inhibited by CYLD overexpression[48]. After stimulating cells with DNA-damage, NEMO is SUMO-ylated and subsequently ubiquitinated on the same residue. Furthermore, both these modifications are required for NF-κB activation after genotoxic stress[56]. The nature of the observed ubiquitination of NEMO after DNA-damage has remained unidentified. Interestingly, NEMO is K6 poly-ubiquitinated by the anti-apoptotic factor cIAP1[57]. However, whether this ubiquitination is identical to the NEMO modification observed after DNA-damage, and how this affects NEMO function are unclear at present.

DNA-repair: a tale of crosslinks, double-strand breaks and ubiquitin

The inherent chemical instability of DNA results in the constant generation of various types of structurally altered bases. The combination of exposure to exogenous DNA damaging agents and the generation of toxic metabolic by-products, such as oxygen radicals, has been estimated to result in more than 10,000 DNA lesions per cell per day[58]. To deal with the scale and variety of DNA lesions,

cells have evolved elaborate DNA repair mechanisms with ubiquitin playing an important role.

Fanconi anemia and monoubiquitination

Failure to repair damaged DNA results in genomic instability with potentially disastrous consequences, including cancer. Fanconi Anemia (FA) is one of many rare hereditary cancer predisposition syndromes that are the result of loss-of-function mutations in critical DNA repair proteins. Other well known examples include Xeroderma Pigmentosum, Ataxia Telangectasia (ATM mutations), Li-Fraumeni (p53 mutations), Seckel- (ATR mutations), Bloom- and Werner syndrome[59]. In addition, mutations associated with hereditary breast cancer have been described in two large DNA-repair proteins called BRCA1 and BRCA2[60].

Thus far, mutations in 11 distinct genes have been implicated in FA[61]. The gene products mutated in these so called complementation groups appear to function in a common pathway or network involved in the repair of DNA inter-strand crosslinks and double strand breaks (DSBs) by homologous recombination (HR)[62]. The exact molecular function of the pathway, however, remains obscure. The majority of the FA proteins cooperate to catalyze the monoubiquitination of a FA protein known as FANCD2 (Fanconi complementation group D2), a critical event in the pathway[63]. The ubiquitin E3 for FANCD2 was recently identified, and is itself a FA protein (FANCL)[64]. FANCL, also known as PHF9, contains a ring finger ubiquitin ligase domain and is part of the "FA core complex", consisting of other FA proteins like FANCA, FANCF, FANCG and the recently identified FANCB[65,66].

Interestingly, many of the proteins mutated in the genomic instability syndromes mentioned, functionally intersect with the FA pathway. For example, phosphorylation of FANCD2 by ATM and ATR is required for its monoubiquitination, and the Bloom protein interacts with the FA core complex[63,67,68]. Monoubiquitination of FANCD2 re-localizes it to the well studied but elusive DNA-damage foci containing BRCA1 and BRCA2. In fact, hypomorphic mutations in BRCA2 constitutes the FANCD1 complementation group[69]. Many questions concerning FANCD2 function remain unanswered. For instance, the anticipated "monoubiquitin receptor" binding FANCD2 on damaged DNA has not been identified. Furthermore, studies on the dynamics of FANCD2 ubiquitination and deubiquitination have been scant. Data presented in this thesis suggest that the deubiquitinating enzyme USP1 removes ubiquitin from FANCD2 once DNA-damage has been repaired at the exit of S phase, possibly contributing to the dissociation and dynamics of DNA-damage foci[70].

BRCA1/BRCA2 ubiquitination

Another ubiquitination event involved in DNA repair by means of HR concerns the E3 heterodimer BRCA1/BARD171. Both proteins contain ring finger E3 domains and are capable of the generation of K6 linked poly-ubiquitin chains in vitro[72-74]. Furthermore, the BRCA1/BARD1 dimer poly-ubiquitinates itself, an event associated with a marked increase in *in vitro* E3 activity[75]. Nonetheless, conclusive studies into the molecular function of BRCA1/BARD1-mediated ubiquitination for DNA repair are missing and relevant in vivo substrates have not been published. A recent report, however, indicates a role for BRCA1 in centrosome duplication though direct regulation of γ-tubulin monoubiquitination[76]. Centrosome amplifications have been linked to aneuploidy, and cells with extra chromosomes were indeed observed upon inhibition of BRCA1 or transfection of a non-ubiquitinatable γ-tubulin mutant. However, a direct role for centrosome amplification through BRCA1 loss in tumorigensis or DNA-repair has not yet been addressed.

Interestingly, BRCA1 has also been found to interact with the deubiquitinating enzyme BAP1. Co-transfection experiments suggest that BAP1 enhances growth inhibition by BRCA1[77]. However, as for BRCA1/BARD1, substrates and functional relevance for DNA-repair remain to be determined.

Also BRCA2 is regulated by ubiquitination and interacts with a DUB. Schoenfeld and colleagues found that the deubiquitinating enzyme USP11 interacts with BRCA2, inhibiting BRCA2 ubiquitination in the absence of DNA-damage[78]. As for BRCA1, these poly-ubiquitin chains do not target the protein for proteasomal desctruction. However, although USP11 inhibition by RNAi or a catalytically inactive mutant did enhance DNA-damage induced cell death in a clonogenic assay, this failed to affect BRCA2 ubiquitination after mitomycin C (MMC, a DNA crosslinking agent) treatment[78]. Thus, a role for USP11 in the regulation of DSB repair remains to be determined. Clearly, ubiquitination and deubiquitination events concerning BRCA1 and BRCA2 continue to puzzle DNA-damage researchers and will likely continue to be a focus for future studies.

The many faces of PCNA

PCNA (proliferating cell nuclear antigen) is an essential protein involved in various DNA-related

processes, including replication and repair (reviewed in[79]). Several exciting studies over the last

Table 3 Examples of human de-ubiquinating enzymes. Indicated are (putative) substrates.

Name	Process/disease	Substrate(s)	Type	Remarks	References
USP9X	Wnt-signaling, endocytosis, estrogen signaling?	β-catenin, epsin	UBP	siRNA confers Tamoxifen resistance (Oosterkamp and Bernards, unpublished)	104,105
USP1	DNA-repair	FANCD2, PCNA?	UBP	Discussed in Chapter 4	70
BAP1	DNA-repair	Unknown	UCH		77
UCH-L1	Parkinson's disease	Unknown	UCH	Also E3	106,107
CYLD	Cylindromatosis, Stress signaling	TRAFs, NEMO?	UBP	Discussed in Chapter 3	47-49
A20	NF-κB signaling	RIP	OTU	Also E3	46
VDUI, VDUII	Hypoxia	Unknown	UBP	RNAi inhibits HIF1-α reporter (Chapter 2)	108,109
USP2	Androgen signaling	Fatty acid synthase	UBP	Over-expressed in prostate cancer	110
USP7 (HAUSP)	Checkpoint signaling	P53, HDM2	UBP	Binds to herpes virus protein Vmw110	111-113
TRE-2	Putative oncogene	Unknown	UBP	RNAi activates E2F reporter	114
UBPY (USP8)	EGF signaling	NRDP1	UBP		115,116
USP10	Ras-signaling?	Unknown	UBP		117
USP16	Chromosome condensation?	Unknown	UBP		118
USP14	Synaptic transmission	Unknown	UBP		119
USP11	DNA-repair	BRCA2	UBP		78
USP26	Spermatogenesis?	Unknown	UBP		120

few years have provided insight into the molecular mechanisms that allow PCNA to perform at the heart of the replisome. PCNA is not only critical for normal (processive) DNA-replication but also required for post-replication repair (PRR). High fidelity polymerases cannot replicate structurally altered DNA due to a stringent requirement for undamaged bases in their active clefts[80]. As a result, replication forks accumulate at sites of damaged DNA, somehow activating PRR. Genetic screens in yeast have identified several genes required for PRR, including the ubiquitin E2 RAD6, the E3s RAD5 and RAD18, and the previously mentioned MMS2/UBC13 E2 heterodimer[81,82]. However, the substrate(s) of these ubiquitin ligases remained unknown for over 25 years. Molecular insight into the role of ubiquitination in PRR came from a landmark paper by Hoege and colleagues studying this process in *S. cerevisiae*[38]. They showed that during PRR, PCNA is either found monoubiquitinated or K63 poly-ubiquitinated on lysine 164. In addition, during normal S phase in the absence of DNA-damaging agents, some PCNA molecules are SUMO-ylated on the same lysine residue[38]. Importantly, the monoubiquitination depends on both RAD6 and RAD18 and the poly-ubiquitination on MMS2/UBC13 and RAD5. Furthermore, these different PCNA modification states are associated with two types of PRR: error-prone translesion synthesis (TS) depends on monoubiquitination, whereas error-free DNA-strand switching relies on K63 poly-ubiquitination. The physiological role of SUMO-ylation of PCNA during S phase remains unclear, since this modification appears to promote mutagenesis even in the absence of DNA-damage[83]. In addition, whereas in human cells monoubiquitinated PCNA can be readily detected upon treatment with methyl-methanesulfonate (MMS, a DNA alkylating agent) or UV irradiation, SUMO-ylation and K63 ubiquitination

of PCNA in mammalian cells have not yet been reported[84]. Recently, RAD18 was found to be a second PRR protein to be modified by monoubiquitin, poly-ubiquitin chains and SUMO[85,86]. Although the exact functions of these modification have not yet been thorougly addressed, mono-ubiqutinated RAD18 appeared to localize primarily to the cytoplasm suggesting an inhibitory role[85].

Translesion synthesis involves the recruitment of specialized DNA polymerases to by-pass structurally altered bases. Likely, these enzymes have evolved to tolerate certain types of DNA damage during S phase, thereby preventing replication fork collapse and generation of (a more serious) DSB[87]. It should be noted, however, that translesion synthesis does not remove the DNA lesion, and thus may continue to pose a threat during subsequent rounds of DNA replication.

A growing number of TS polymerases have been identified and although they share little sequence similarity with normal high-fidelity DNA polymerases, crystal structures show that they share the characteristic palm, finger and thumb domains[88]. TS polymerases, however, lack proofreading exonuclease activity and display low fidelity on undamaged DNA. Nonetheless, to designate these polymerases as low fidelity DNA polymerases is not correct. TS polymerases do display high genetic fidelity on specific types of damaged bases, inserting the correct base that would normally pair with the undamaged version. For instance, when replicating undamaged DNA, the TS polymerase polη is sloppy, inserting incorrect bases approximately 2000 times more often than DNA polε[88]. However, when replicating a T-T cyclobutane pyrimidine dimer (a lesion induced by UV irradiation), polη is capable of inserting the genetically correct A opposite the damaged T[89].

In yeast, genetic studies indicate that the polymerases η and ζ act downstream of monoubiquitinated PCNA to facilitate translesion synthesis[38]. Indeed, studies in mammalian cells show that monoubiquitinated PCNA specifically interacts with polη in DNA-damage foci following UV–irradiation[84,90,91]. Furthermore, this interaction depends on a CUE-like ubiquitin interaction domain in the "finger" of polη, suggesting direct binding. As for FANCD2, many issues concerning regulation of PCNA ubiquitin (-like) modifications remain to be addressed. For example, the mechanism of stalled replication fork sensing and the signals required for activating the ubiquitin ligases upstream of PCNA have not been characterized. Also studies into the mechanisms downstream of PCNA that would allow switching back to normal DNA-replication have not been reported. However, since the interaction of PCNA with TS polymerases appears to be regulated by monoubiquitin, it is likely that this involves deubiquitination.

Deubiquitining enzymes: All in the family

As discussed, besides protein ubiquitination, deubiquitination is emerging as an important regulatory mechanism (also see Table 3). All members of the deubiquitinating enzyme (DUB) family are cysteine proteases cleaving the peptide bond between the C-terminal glycine of ubiquitin and the subsequent lysine[92,93]. Untill recently, the family was subdivided into type I (also called UCH) and type II (also known as UBP) ubiquitin proteases. However, the discovery of OTU-domain ubiqutin proteases, containing a distinct three-dimensional protease architecture, has added a third class of DUBs[93,94]. Finally, the JAM (Jab1/MPN domain-associated metalloisopeptidase) motif containing protein Rpn11 has been shown to deubiquitinate proteins entering the proteasome[26]. Rpn11 is the only known ubiquitin protease that does not rely on a cysteine in its active site. For this reason, Rpn11 is generally not considered a DUB family member and referred to as a "cryptic" DUB. Structural and functional studies indicate that UCHs are small (20-30 kDa) papain-like proteases, that cleave ubiquitin from short polypeptides (up to 20 residues) with relatively low specificity[95]. Only four human UCHs have been identified, including the previously mentioned BAP1. The bulk of DUB family members is made up by UBPs, of which approximately 55 have been identified in the human genome[70]. UBPs can be readily identified based on their conserved catalytic domain which contains six short conserved regions encompassing 300 to 500 amino acids[96]. Three of these regions surround the cysteine, histidine, and aspartic acid residues forming the "catalytic triad" of the enzyme[97]. The function of the three other regions has not been determined but may involve ubiquitin binding. However, a mechanism for the recognition of specific ubiquitin chains by UBPs, for example K63 vs. K48, has not been proposed. Indeed, some of these putative ubiquitin de-conjugating enzymes may cleave other ubiquitin-like moieties. For instance, it was shown that the DUB family member USP18 can specifically cleave ISG15 from its targets[98]. UBPs differ greatly in size, varying from approximately 30-300 kDa, and have occasional large insertions in their catalytic domain. Besides a subclass of UBPs that contain additional ubiquitin binding motifs in their N- and C-termini (also see Table 1), UBPs share little sequence homology,

suggesting distinct functions. Like ubiquitin ligases, mammalian UBPs have been implicated in numerous processes but for the majority of DUBs, substrates have not been identified (see Table 3). In addition, studies in yeast have implicated DUBs in DNA silencing and transcriptional activation, processes that in mammalian cells have not yet been reported to involve deubiquitination[99-103].

Concluding remarks

A large number of studies over the last decade have uncovered an unanticipated diversity of protein regulation by ubiquitin and ubiquitin-like molecules. Clearly, nature has adopted the flexibility and versatility of ubiquitin (like) molecules in almost all aspects of cell biology. Although our knowledge on protein modification by ubiquitin has expanded in an almost exponential fashion, many issues concerning ubiquitination remain poorly understood, some of which were pointed out in this introduction. The following three chapters deal with one of those aspects: the de-conjugation of ubiquitin by DUBs. Using RNAi-based technology we have identified DUBs involved in a number of processes including hypoxia signaling (chapter 2), NF-κB signaling (chapter 3), and DNA-repair (chapter 4). Possibly, similar genetic studies will further contribute to the functional annotation of DUBs and identify DUB substrates in the future.

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